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Transcriptome changes in a colon adenocarcinoma cell line in response to photochemical treatment as used in photochemical internalisation (PCI)

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Abstract The photochemical internalisation (PCI) technology liberates endocytosed macromolecules like transgenes from endocytic vesicles in response to photochemical treatment. Thereby PCI improves gene transfection and is suggested for use in gene therapy. It has been proposed that PCI might also stimulate transcription of internalised transgenes, especially if they are controlled by photochemically inducible promoters (transcriptional targeting). In order to identify inducible promoters, and to evaluate the treatments influence on cellular transcriptional activity, the effect of the photochemical treatment as used in PCI (with the photosensitizer disulfonated meso-tetraphenylporphin followed by illumination) on gene transcription in WiDr adenocarcinoma cells was evaluated using microarrays. The expression of 390 genes were identified significantly changed (89% were up-regulated), of which genes associated with DNA binding and transcriptional functions were the most represented. This may be important for the expression of a photochemically internalised transgene under a specific promoter control. Real-time PCR verified photochemical up-regulation of the HSP family genes, as well as down-regulation of EGR-1 at 2–10 h post-treatment, suggesting that the HSP (particularly HSP70), in addition to the microarray-identified metallothioneins, but not the EGR-1 promoters, could be relevant promoter candidates for transcriptional targeting via PCI. The resulting overview of gene expression changes in WiDr cells exposed to the PCI-relevant photochemical treatment also provide a basis for the design of new PCI-based strategies with respect of transcriptional targeting.

Keywords: Gene therapy; Gene expression profile; Microarray; Photochemical internalisation; Photodynamic therapy; Transcriptional targeting

1. Introduction

Most macromolecular drugs meant to act intracellular enter cells via endocytosis, and are trapped in the endocytic vesicles, from where they are unable to escape to perform a therapeutic function. Liberation of such macromolecules into the cytosol can be achieved by the photochemical disruption of the membranes of the endocytic vesicles [1]. Use of endosome-localising photosensitizers activated by illumination with visible light (i.e. photochemical treatment) generates singlet oxygen, which induces permeabilisation of the vesicular membranes. This results in release of the vesicular content in photochemically treated cells [2], but not in cells unexposed to light. This concept has been named photochemical internalisation (PCI) [1] and it is based on photosensitizers with photochemical properties similar to those utilized in photodynamic therapy (PDT) of cancer. PCI has been successfully applied pre-clinically for delivery of chemotherapeutic agents [3], protein toxins [4,5] and peptide nucleic acids (PNA) [6], enhancing their anticancer activity in vitro and in vivo at specific sites, i.e. photochemically treated tumors. In gene therapy, PCI has resulted in enhanced expression of foreign genes (transgenes) carried by viral [7–9] and nonviral vectors [10–13]. Endosomal release of the transgene is recognised to be the main reason for this enhancement in PCI-treated cells [9,10]. However, recently it has been proposed that PCI-treatment could stimulate also transcription of the liberated transgene, especially if this transgene is controlled by a photochemically inducible promoter [14]. Such transcriptional targeting would enhance the specificity of PCI-based gene therapy. The potential of this idea has been tested employing a promoter of the Heat Shock Protein gene HSP70 [14], known usually to be induced by various stressors such as PDT [15]. Though PCI-dependent activation of the HSP70 promoter-controlled transgene has been demonstrated, the enhancement was moderate [14]. Therefore, a more thorough analysis of the photochemical influence on a cellular transcriptional apparatus and identification of PCI-inducible promoters was needed for a rational utilisation of PCI in transcriptional targeting. Moreover, recently it has been shown that the PCI effect on transgene expression controlled by the cytomegalovirus (CMV) promoter is more pronounced at the mRNA level than at the protein level [9]. This suggests that PCI might influence the transcription positively, and/or the translation machinery of the cell negatively. So far, the cellular

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Abbreviations: CMV, cytomegalovirus; FDR, false discovery rate; HSP, heat shock protein; MT, metallothionein; PCI, photochemical internalisation; PDT, photodynamic therapy; PNA, peptide nucleic acids; SAM, significance analysis of microarrays; $TPPS_{2a}$, disulfonated meso-tetraphenylporphin

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responses to photochemical treatment as used in PCI have been little investigated, despite that the efficiency of PCI-based therapies might be affected by changes in different cellular processes. For example, PCI might modulate expression of genes encoding proteins that are involved in transcription, translation and/or the function of the internalised transgene. Therefore, characterisation of changes related to various cellular functions is important for understanding and further development of PCI-based strategies.

In the present study, we addressed the problem of the cellular response to the photochemical treatment as used in PCI by profiling gene expression. To date very limited data on genome-wide transcriptional changes following photochemical treatment are available. Most of these studies aimed to reveal the molecular mechanisms of PDT-mediated cell killing, and employed photosensitizers not relevant for PCI [16,17]. Since the molecular response to photochemical treatment varies significantly depending on the photosensitizer's localisation (i.e. where singlet oxygen is generated) [18], for PCI purposes it was of importance to characterise changes associated with the PCI-relevant treatment, i.e. with a photosensitizer like disulfonated meso-tetraphenylporphin ($TPPS_{2a}$), localised to the membranes of endocytic vesicles.

The aim of the present study was to identify genes that were transcriptionally regulated by a PCI-relevant photochemical treatment as detected using microarrays. This would lead to the indentification of the photochemically inducible promoters that could be combined with PCI to mediate transcriptional targeting. Also, this would indicate which cellular functions are affected following PCI.

2. Materials and methods

2.1. Photochemical treatment

The human adenocarcinoma cell line WiDr (ATCC, CCL-218) was cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (Sigma, MO, USA) at 37 °C in a 5% CO_2 atmosphere. For photochemical treatment, the cells were incubated overnight in a medium containing 0.7 μg/ml the photosensitizer $TPPS_{2a}$ (Frontier Scientific, Logan, UT), chased for 4 h in $TPPS_{2a}$-free medium and exposed to blue light (LumiSource® 4 * 18 W Osram L 18/67 Blue (PCI Biotech AS, Norway)) with an irradiance of 11.7 mW/cm^2. The light dose was 0.7 J/cm^2, which corresponded to 60 s illumination and led to a cell survival of ~50% (LD50) as measured by the MTT-assay one day after the treatment.

2.2. RNA preparation and labeling for arrays

Total RNA was isolated 3 h after photochemical treatment using Trizol Reagent (Invitrogen, Carlsbad, CA). The total RNA from control cells, not treated with the photosensitizer, but exposed only to blue light, was used as a reference. cDNA was synthesized from 40 μg of total RNA by an oligo(dT)-primed reverse transcriptase reaction and labeled with Cy3 and Cy5 using an indirect aminoallyl FairPlay™ (Stratagene, La Jolla, CA) microarray labeling kit, according to the manufacturer's recommendations. Lucidea™ Universal ScoreCard (Amersham Biosciences) spike mRNAs were added to the samples prior to cDNA synthesis. Labeled Cy3- and Cy5-cDNA was up-concentrated to 10 μl using Microcon YM-30 columns (Amicon, Millipore Corporation, Bedford, MA).

2.3. cDNA microarrays and hybridisations

The microarrays were produced in house using a Micro Grid II robotic printer (Bio Robotics, Cambridge, UK). The targets (13.000 unique I.M.A.G.E. cDNA clones from ResGen 40 k set) were spotted on aminosilane-coated UltraGAPS slides (Corning Life Sciences, Corning, NY). For details on the arrays, we refer to: http://www.mikromatrise.no. The hybridization volume of 110 μl consisted of: 8–10 μl of each of the labelled probes, 16 μg poly A (Amersham Biosciences), 15 μg human Cot-1 DNA (Invitrogen) and 85–90 μl of Microarray Hybridization Buffer #1 from Ambion (Austin, TX). The final mix was heated for 2 min at 100 °C and after spinning applied to a microarray. A hybridization station (Genomic Solutions, Inc., Ann Arbor, MI) was used for hybridization and wash (the details can be found in http://www.mikromatrise.no).

2.4. Data preparation and analysis

The microarray slides were scanned using an Agilent Microarray scanner (Agilent Biotechnologies, Palo Alto, CA). The image quantifications were performed by the software GenePix 4000B (Axon Instruments, Union City, CA). The DNA spots were captured by the adaptive circle segmentation method. The images (TIFF files) and extracted raw data (GRP files) were stored in a BASE 1.2.15 database. The spots that were technically flawed or flagged automatically by the GenePix software, as well as spots with a diameter less than 60 mm, were removed from the data of each microarray. Background-subtracted intensities less than two times the standard deviation (S.D.) of the local background in one channel were assigned this value to avoid zero or negative values in the ratio calculations. Weak spots with background-subtracted intensity less than two times the S.D. of the local background in both channels were excluded. Systematic errors were corrected by normalizing the data, using the locally weighted scatterplot smoother (LOWESS) procedure. The genes were preserved if the values were experimentally obtained in three or more arrays in the experimental matrix.

The significantly altered genes were identified by Significance Analysis of Microarrays (SAM) [19] by the use of the TIGR Multiexperiment Viewer (MeV) (http://www.tm4.org/mev.html). For functional classification of the genes, UniProt Knowledgebase keywords (Release 50.4 of July 25, 2006) and the KEGG Pathway Database (July 19, 2006) were used. Functional classification was performed by using the Database for Annotation, Visualization and Integrated Discovery (DAVID, release 2.1) [20] on line tools. The gene-enrichment of functional categories was measured by determining the number of genes belonging to the functional group in the list of modulated genes weighed against the total number of analysed genes on arrays (background) through the use of the Fisher Exact test.

2.5. Real-time PCR

Total RNA was isolated from the control cells (as in microarray experiments) and from the photochemically treated cells at 2 h, 4 h and 10 h post-treatment by the use of Trizol Reagent (Invitrogen). The cDNA was synthesized from 1 μg DNase I-treated RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). The cDNA solution was diluted 1:3 or 1:6 with nuclease-free water. Real-time PCR was performed using an iQ SYBR Green supermix (Bio-Rad) and specific primer pairs for the selected genes (specified in Supplementary Table 1 and Fig. 2). Primers (specified in Supplementary Table 1) were designed using the software Primer Express (Applied Biosystems, Foster City, CA). For each sample, the following mix (meant for two parallels) was prepared: 10 μl cDNA, 30 μl iQ SYBRGreen Supermix, 300 nM of each primer (MWG Biotech AG, Germany) in and nuclease-free water to a final volume of 60 μl. Aliquots of 25 μl were distributed in two wells on the PCR plate. Real-time PCR reactions were run on an iCycler (Bio-Rad) with the following amplification protocol: 3 min initial denaturation at 95 °C, 50 cycles of 10 s denaturation at 95 °C and 35 s annealing/extension at 60 °C. A final melt curve analysis was included to verify that one specific product was obtained in each reaction.

The fold changes in the relative gene expression were calculated using the Gene Expression Macro, version 1.1 (Bio-Rad). Calculations were based on the ΔΔCt method, in which the threshold cycle number (Ct) for each studied gene in each sample were normalised to the Ct-value of the reference gene in the same sample. As a reference gene, we used human acidicribosomal phosphoprotein P0 (RPLPO), which was not affected by the photochemical treatment. The normalized values (ΔCt) in the control sample were given a value of 1, and the fold-change (ΔΔCt) between the photochemically treated samples and the controls were calculated accordingly.

3. Results and discussion

To reveal the influence of a PCI-relevant photochemical treatment on gene expression, the cells were treated with the photosensitizer $TPPS_{2a}$ followed by illumination. A photochemical dose corresponding to ~LD50 was employed, based on previous studies demonstrating this dose to lead to an optimal PCI effect on transgene expression [21] and to modulate cellular responses, i.e. induce stress-response [14]. A time point – 3 h post-treatment – was chosen for the microarray experiments, based on the assumption that this is sufficient for the liberated transgenes to reach the nucleus. Therefore, characterisation of the transcriptome at this time point was expected to reveal the changes that could be directly implicated in transcription of these transgenes. It has been reported by others that nuclear localisation of a foreign gene may be observed as early as 1 h post-treatment (for adenoviral vectors and one lipidic vector [22]), though usually nuclear transport is a slower, time-dependent process, especially for nonviral systems [23]. Moreover, nuclear transport of a transgene after PCI can be slowed down due to e.g. photochemical effects on a cell cycle [24]. Therefore, we assumed that 3 h represented the earliest relevant time point for screening of PCI inducible promoters, and for identification of modulated genes with transcription-associated functions.

We used the WiDr cell line that has already been used for PCI-studies, both in gene – [7–9,14], protein – [4] and chemotherapy [3] studies, demonstrating efficient responses to PCI. Moreover, PCI in WiDr cells resulted in: (i) a lower-than-expected effect employing a promoter from the stress-response family HSP70 [14] and ii) a higher effect at the mRNA than at the protein level of a photochemically internalised transgene driven by the CMV promoter, indicating a PCI influence on cellular transcription/translation on a general basis [9]. Therefore, to understand the molecular basis behind these observations, it was of importance to characterise the response to photochemical treatment in these cells.

3.1. Characterisation of photochemically induced changes in gene expression

To identify genes with significantly modulated expression in photochemically treated cells compared to the control cells, we used the one-class SAM method that assigns a score to each gene on the basis of change in gene expression relative to the S.D. of repeated measurements [19]. SAM uses permutations of the repeated measurements to estimate the percentage of genes identified by chance, i.e. the false discovery rate (FDR). In our study, SAM identified 390 significantly modulated genes that changed at least 1.4-fold with an estimated FDR of less than 5% (here FDR is reported as FDR90% (percentile), i.e. we claim with 90% confidence that there are no more then 5% of false discovered genes in the significantly modulated gene list). The identified genes were clustered by hierarchical clustering, sorting the genes based on similar transcriptional changes (Fig. 1). As can be seen, the up-regulated genes dominated, constituting 88.5% of the modulated genes. Importantly, the dye-swap experiment was clustered with the non-swapped experiments (Fig. 1), indicating that the results represent biological and not methodological variations.

Usually, the level of gene transcription reflects the activity of the corresponding promoters [25]. A corollary to this is that

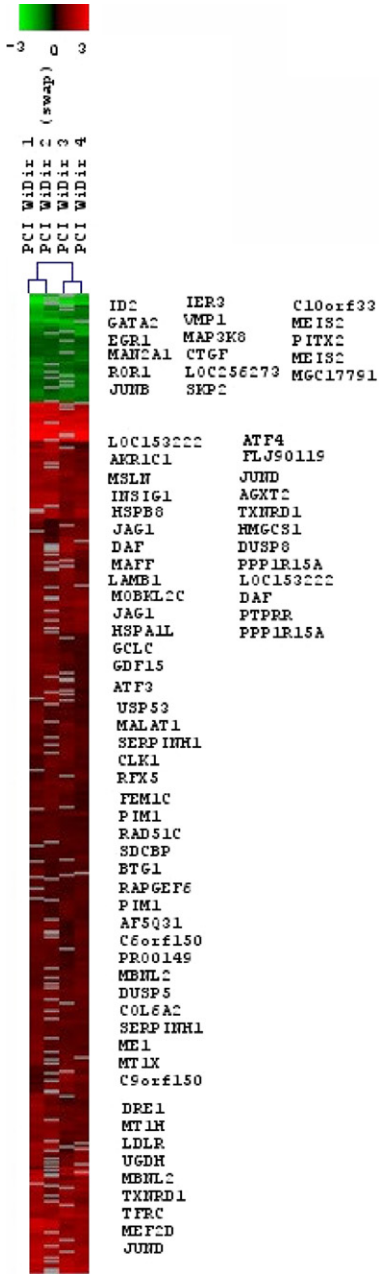


Fig. 1. Changes in gene expression in WiDr cells exposed to the PCI-relevant photochemical treatment. Differentially expressed genes were identified by one-class SAM with an estimated <5% of FDR (90 percentile). The analysis was based on the data from three biological experiments and four hybridizations (including one dye-swap). The diagram illustrates the results of hierarchical clustering, showing a subset of genes that are suppressed and induced. The gene symbols of some obviously down- and up-regulated genes are presented as an illustration. Horizontal rows represent genes, and columns represent the arrays corresponding to the number of the experiments. The fold-changes of expression (presented on the $\log_2$-based scale) are color coded as shown in the bar above, and grey elements represent missing/quality filtered values.

the up-regulated genes indicate which promoters that are photochemically induced and may be combined with PCI for transcriptional targeting. The cluster of up-regulated genes comprised, among others, a group of stress-response genes from the HSP-family, like HSPA1L (HSP70-HOM),

SERPINH1 (HSP47) and HSPB8 (HSP22) (Fig. 1), corresponding well to the general notion that PDT induces HSPs [15]. Among other stress-related genes in the up-regulated gene cluster was a ~3-fold up-regulated group of metallothioneins (MT) (e.g. MT1X and MT1H) that, like HSPs, are induced in response to stress, e.g. oxidative stress, for cellular protection [26].

Conversely, down-regulated genes should reveal promoters that would not be optimal in combination with PCI. The cluster of down-regulated genes included, among others, the gene encoding the early growth response 1 (EGR-1) transcription factor that is known to be significantly up-regulated by ionising radiation [27].

Of interest, genes that inhibit transcription, e.g. inhibitor of DNA binding ID2 or transcription factor JUNB (which antagonises the potent transcriptional activator JUN) appeared in the cluster of down-regulated genes, suggesting that the photochemical treatment itself does not hamper the general transcriptional activity of a cell.

3.2. Functional classification

The differentially expressed genes were also classified based on the cellular functions involving these genes. Such classification indicates the cellular functions that are likely to be affected in the cells exposed to PCI. Annotations for functional categories were found for 91% (UniProt Knowledgebase keywords) and 28% (KEGG Pathways database) of the modulated genes. Table 1 presents the functional groups and pathways that, as the differentially expressed genes indicate, were affected in the photochemically treated cells. As can be seen, the genes associated with nuclear proteins and transcription, transcription regulation and DNA binding functions were significantly represented ($P < 0.05$) in the list of differentially expressed genes. This suggests that the processes occurring in the nucleus and related to transcription are affected in cells exposed to the PCI-relevant photochemical treatment. This means that transcription of a foreign gene, internalised via PCI, may well be affected by the photochemical influence on the cell's transcriptional apparatus.

Besides the genes directly associated with transcription, we identified that ~4% ($P = 0.056$) of the affected genes could be associated with cell cycle functions. Photochemical modulation of cell cycling might indirectly influence the expression of a foreign gene, especially delivered by nonviral vectors, since its transport to the nucleus requires transition through mitosis [28]. On the other hand, expression of a foreign gene delivered by adenovirus and PCI might be not dependent on the cell cycling, since transgene-adenovirus nuclear transport is independent from the mitotic events [28].

Table 1 also reveals the photochemical influence on several signalling pathways like TGF-β, MAPK and WNT. Regulation of signalling pathways might have broad consequences for various cellular functions that might influence activities of the internalised macromolecules, and these issues remain to be elucidated.

Supplementary Table 2 extends the information presented in Table 1 and specifies the genes from the particular functional categories and presents fold-changes for these genes. A number of genes encoding transcription factors were identified, including activating protein-1 (AP-1) transcription factors (mostly up-regulated), as well as genes (up-regulated) related to the Nuclear Factor κB (NF-κB) pathway (see Supplementary Table 3). This is in agreement with Luna et al. [29] and Piette et al. [30], who demonstrated PDT-mediated induction of AP-1 (Jun, Fos) and NF-κB, respectively. Activation of AP-1 and NF-κB might trigger transcription from the promoters carrying the binding sites for these transcription factors. In this respect, one might consider the stimulation of transcription from the well-known CMV promoter, generally considered as non-inducible and employed in the majority of the PCI-based gene therapy studies [7–10,14]. The CMV promoter has AP-1 and NF-κB binding sites, and it has been shown before that AP-1 and NF-κB are involved in CMV stimulation [31]. Thus, the microarray-based data strengthen the theoretical possibility that PCI might also influence transcription from the CMV promoter. This possibility has been supported by the demonstration of a ~2-fold photochemical stimulation of the genome-integrated transgene controlled by the CMV promoter in WiDr cells (Prasmickaite, unpublished results). This is also in line with the data of Engesæter et al. [9], who showed that the PCI effect on CMV-controlled transgene expression is pronounced more at the mRNA than at the protein level, suggesting potentiated transcription, or enhanced stability of mRNA. However, it should be stressed that Engesæter et al. [9] clearly demonstrated that PCI affects mainly the endocytic vesicles,

Table 1
Enriched Functional Categories and KEGG pathways in the list of differentially expressed genes[a]

Term[b]	Count	%[c]	*P*-Value
UniProt Knowledgebase			
Nuclear protein	95	24.4	0.016
Metal-binding	67	17.2	0.037
DNA-binding	59	15.1	0.000056
Transcription regulation	58	14.9	0.00035
Transcription	56	14.4	0.0014
Zinc	55	14.1	0.021
Activator	22	5.6	0.0038
Cell cycle	17	4.4	0.056
Proto-oncogene	14	3.6	0.028
Ank repeat	11	2.8	0.017
Protein phosphatase	8	2.1	0.098
Methylation	6	1.5	0.047
Heat shock	5	1.3	0.0073
Copper	5	1.3	0.057
Metal-thiolate cluster	5	1.3	0.000024
Cadmium	5	1.3	0.000024
Steroid biosynthesis	5	1.3	0.019
KEGG_pathway			
MAPK signaling	17	4.4	0.018
WNT signaling	12	3.1	0.025
TGF-β signaling	8	2.1	0.045
T cell receptor signaling	8	2.1	0.049
Adherens junction	8	2.1	0.023
Biosynthesis of steroids	4	1.0	0.018
Terpenoid biosynthesis	3	0.8	0.021

[a]Genes with significantly changed expression following the PCI-relevant photochemical treatment were identified by the SAM analysis with an estimated FDR of less than 5% (90 percentile, i.e. 90% confidence that there are no more then 5% of false discovered genes in the significantly modulated gene list).
[b]Functional classification was based on UniProt Knowledgebase keywords and KEGG pathway Database.
[c]The gene-enrichment of functional categories was measured by determining the number of genes belonging to the functional group in the list of modulated genes, weighed against the total number of analysed genes on arrays (background) using the Fisher Exact test.

meaning that its influence on transcription from the CMV promoter could be relatively moderate. Nevertheless, photochemical stimulation of transcription from the CMV promoter would strengthen the concept of exploiting PCI as a switch for transcriptional targeting employing appropriate promoters.

An interesting finding is the significant up-regulation of the metal-thiolate functional category, comprising different metallothioneins (Supplementary Table 2). The photochemical influence on the MT genes/promoters has so far not been well characterised, although the oxidative-stress mediated induction of MTs has been reported [26]. One may speculate that over-expression of MTs that act as antioxidant agents can scavenge the reactive oxygen species and might have consequences for the progression of photochemical reactions, and consequently for the effectiveness of PDT. On the other hand, photochemically stimulated MT promoters might find an application for transcriptional targeting via PCI. The employment of the MT promoter for controlling transgene expression in response to electric fields has already been demonstrated in vivo [32], indicating the potential for MT promoter-based regulated gene expression systems.

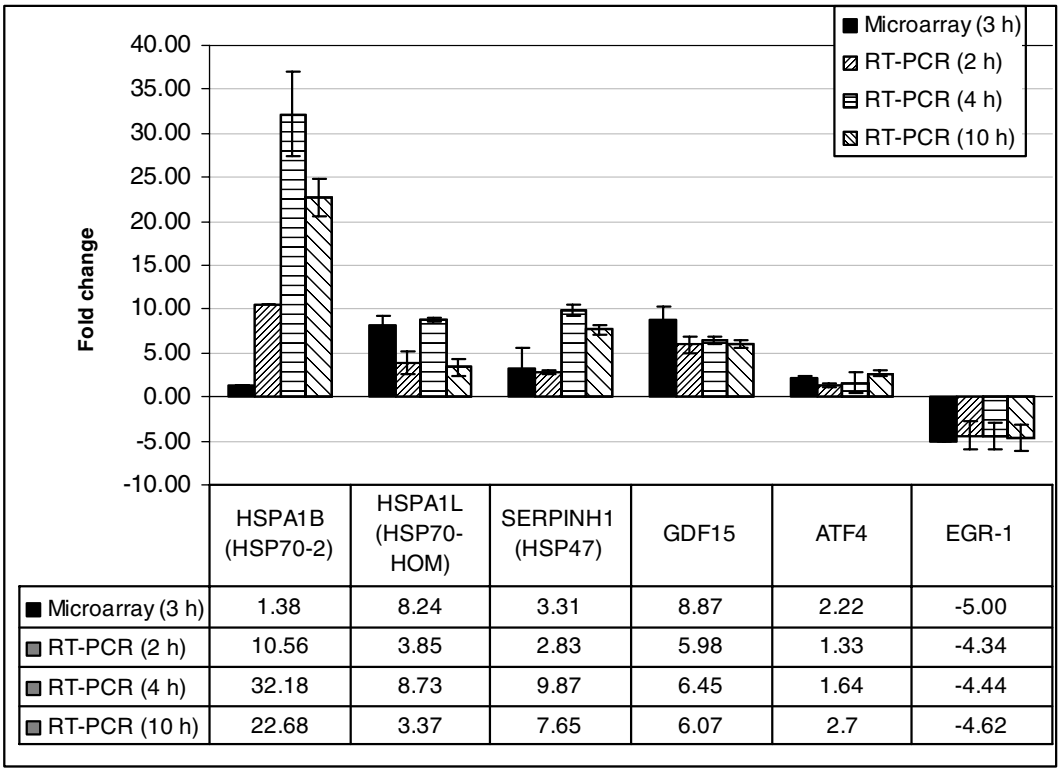
3.3. Photochemical effect on HSP family genes

The heat shock functional category, including the HSP genes (Supplementary Table 2), was analysed in more detail as shown in Table 2. Characterisation of the photochemical effect on these genes might be of interest for cancer therapy, and specifically for gene therapy and PDT, for several reasons: (i) the possible use of the HSP promoters for transcriptional targeting when combined with the cancer targeting method PCI [14]; (ii) HSPs role in tumorigenesis, and in resistance to senescence/apoptosis [33,34]. As can be seen, many HSP genes were photochemically induced, though at different levels. HSP70-HOM was identified by the microarray analysis as the most highly induced (~8-fold). However, it should be emphasized that the real-time PCR data (Fig. 2) identified another HSP70 gene, HSPA1B (HSP70-2), as the most up-regulated (up to 32-fold), contradicting the microarray data. HSP70-2, -1 and -HOM are highly homologous genes, forming HSP70 (the major HSP), although their response to stress might differ. It has been shown that nearly identical HSP70-1 and -2, often collectively referred to as HSP70i, contains the Heat Shock Element (HSE) in their promoter and, therefore is considered the major stress inducible HSP70. HSP70-HOM contains the cAMP Responsive Element (CRE), but lacks HSE and, hence, generally is non-inducible [35], although it responded to the photochemical treatment as shown in Table 2 and Fig. 2. Identification of the transcription factors and the regulatory elements responsible for the photochemical activation of a promoter will be an important task for future research.

Table 2
The heat shock protein (HSP) family genes analysed on arrays

Gene group/gene name	Gene symbol	Aliases	Locus link	Fold change[a]
HSP genes Hsp100				
Heat shock 105 kDa/110 kDa protein 1	HSPH1	HSP105A	10808	↑1.7 ± 0.2
Heat shock 70 kDa protein 4	HSPA4	APG-2	3308	1.3 ± 0.1
Hsp 90				
TNF receptor-associated protein 1	TRAP1	HSP75	10131	0.9 ± 0.1
Hsp70				
Heat shock 70 kDa protein 1-like	HSPA1L	HSP70-HOM	3305	↑↑↑8.2 ± 2.2
Stress-induced-phosphoprotein 1 (Hsp70/Hsp90-organizing prot.)	STIP1	HOP	10963	↑1.7 ± 0.2
Heat shock 70 kDa protein 8	HSPA8	HSC70	3312	↑1.6 ± 0.1
Heat shock 70 kDa protein 1A	HSPA1A	HSP70-1	3303	1.2 ± 0.3[b]
Heat shock 70 kDa protein 1B	HSPA1B	HSP70-2	3304	1.4 ± 0.0[b]
Heat shock 70 kDa protein 9B (mortalin-2)	HSPA9B	GRP75	3313	1.4 ± 0.0
Heat shock 70 kDa protein 12A	HSPA12A		259217	1.1 ± 0.1
Heat shock 70 kDa protein 14	HSPA14	HSP70-4	51182	0.9 ± 0.1
Hsp 40				
Serine (or cysteine) proteinase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)	SERPINH1	HSP47	871	↑↑3.3 ± 0.9
DnaJ (Hsp40) homolog, subfamily A, member 1	DNAJA1		3301	↑↑2.7 ± 0.3
DnaJ (Hsp40) homolog, subfamily B, member 9	DNAJB9		4189	↑↑2.3 ± 0.3
Small Hsp				
Heat shock 22 kDa protein 8	HSPB8	HSP22	26353	↑↑↑5.0 ± 1.5
Heat shock protein, alpha-crystallin-related, B6	HSPB6	Hsp20	126393	1.0 ± 0.3
HSPB (heat shock 27 kDa) associated protein 1	HSPBAP1		79663	1.3 ± 0.2
Heat shock 27 kDa protein 2	HSPB2	HSP27	3316	1.1 ± 0.1
Other related to Hsp				
Heat shock factor binding protein 1	HSBP1		3281	1.0 ± 0.1
Heat shock transcription factor 2	HSF2		3298	1.3 ± 0.3
Hsp70-interacting protein	HSPBP1		23640	1.0 ± 0.2

[a]Fold change determined by signal intensity in photochemically treated samples divided by signal intensity in control samples. Fold change is presented as the mean ± S.D. of four parallel experiments. Arrows indicate significant fold-changes.
[b]The results have not been confirmed by real-time PCR, which indicated a significant up-regulation (see Fig. 2).



	HSPA1B (HSP70-2)	HSPA1L (HSP70-HOM)	SERPINH1 (HSP47)	GDF15	ATF4	EGR-1
Microarray (3 h)	1.38	8.24	3.31	8.87	2.22	-5.00
RT-PCR (2 h)	10.56	3.85	2.83	5.98	1.33	-4.34
RT-PCR (4 h)	32.18	8.73	9.87	6.45	1.64	-4.44
RT-PCR (10 h)	22.68	3.37	7.65	6.07	2.7	-4.62

Fig. 2. Real-time PCR-based validation of the photochemical effect on gene expression in WiDr cells exposed to the PCI-relevant photochemical treatment. Six selected genes – HSPA1B (HSP70-2), HSPA1L (HSP70-HOM), SERPINH1 (HSP47), GDF15, ATF4 and EGR-1 – were assessed for expression at mRNA level at 2 h, 4 h and 10 h after photochemical treatment by real-time PCR, using the housekeeping RPLPO gene as a reference gene. The fold change in gene expression in photochemically treated samples compared to non-treated controls was calculated and presented as the mean ± S.D. of three real-time PCR runs performed in duplicates. The fold-changes obtained by the microarray analysis (black-bars) are presented for comparison.

3.4. Validation of the photochemical effect on gene expression by real-time PCR

To verify the photochemical effect on expression of genes identified by the microarray analysis, real-time PCR was performed on RNA samples prepared at 2 h, 4 h and 10 h after PCI treatment. The different time points were chosen for real-time PCR to cover the 3 h time-point used for microarrays and, at the same time, to evaluate the earlier and the later photochemical effects on the selected genes. It is generally accepted that real-time PCR provides a relatively reliable and methodologically independent measurement, and thus additional information regarding the photochemical effect on gene expression initially characterised by microarrays.

The following genes were selected for validation of the photochemical influence: the significantly up-regulated genes like HSP70-HOM, HSP47, GDF15, ATF4; the significantly down-regulated EGR-1; and the insignificantly affected (~1.4-fold up-regulation) HSP70-2. The real-time PCR data generally agreed with the microarray data, demonstrating comparable levels of photochemical stimulation/repression of selected genes (Fig. 2). The only exception was the HSP70-2 gene (Fig. 2), which was the most up-regulated according to the real-time PCR data (using two different primer sets). This up-regulation corresponds well to our earlier published Northern blot data indicating photochemical induction of HSP70i [14]. Taken together, we conclude that the HSP promoters, particularly HSP70, respond to our photochemical treatment, resulting in enhanced transcription of the corresponding HSP genes.

It should be noted that the level of photochemical stimulation of the HSP-family genes/promoters depended on the time-point after treatment (Fig. 2). The 4 h-time point led to the highest HSP up-regulation among the three time points tested by real-time PCR, and 10 h showed reduced up-regulation. This suggests that success of transcriptional targeting via PCI might depend on the overlap-in-time of two events: the photochemical stimulation of the employed promoter and the transgenes nuclear localisation (i.e. ''readiness'' for transcription). One may expect that the optimal promoter for transcriptional targeting via PCI would be the one that is stimulated at the right moment, and not necessarily the one that is stimulated the most. So far, no studies have been performed to reveal these issues. The lack of such overlap could be one of the reasons for a lower-than-expected effect in the previous study employing one of the HSP70 promoters in combination with PCI [14]. However, another reason could be that the rat HSP70.1 promoter employed in that study [14] did not respond in the same way as the human HSP70-2 identified in the present study.

Real-time PCR data also confirmed the significant and prolonged down-regulation of EGR-1 (Fig. 2). Thus, both the microarray- and the PCR-data suggest that the EGR-1 promoter does not seem to be appropriate for combination with PCI for driving expression of an internalised transgene. EGR-1 induction via stimulation of the promoter in response to ionising radiation is a well-known phenomenon, and the EGR-1 promoter has been successfully employed in gene ther-

apy for transcriptional targeting when combined with radiotherapy [27]. The PDT influence on EGR-1 has already been studied by others employing another photosensitizer [29], and it has been demonstrated a rapid induction of EGR-1 following PDT, although EGR-1 mRNA decayed rapidly (within 1.5 h), indicating a very short-lasting photochemical induction.

We also confirmed the up-regulation of the activating transcription factor 4 (ATF4) (Fig. 2), which mediates recovery from the stress-induced shut-off of translation [36]. Induction of ATF4 might imply a temporal inhibition of the translational machinery of a cell after PCI. This would be in line with Engesæters data, showing the lower PCI effect at the protein than at the mRNA level of the internalised transgene [9]. In this respect, PCI of PNA or siRNA, that do not need the translation step to perform a therapeutic function, should lead to higher therapeutic improvements via PCI.

3.5. Conclusion

This study is the first attempt to characterise transcriptomic changes induced by photochemical treatment as used in PCI, with the aim to extend our knowledge regarding the PCI mechanisms of action, particularly in respect to transcriptional targeting. Understanding of the photochemical effect on expression of genes, like the ones associated with the transcription/translation functions, might help to foresee how the photochemically internalised transgene (as a plasmid or in an adenoviral genome) will be processed. Consequently, this information might be employed for the design of new PCI-based gene therapy approaches, such as through stimulation of promoters and transcription via the PCI stimulus. Further development of the PCI-based targeting should include the application of alternative promoters, for example the ones from the HSP and the MT gene families, and cellular mechanisms related to PCI-induced cellular response, as demonstrated by the information gained through the microarray analysis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.09.028.

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